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Design of a mode of delivery for macrobead transplantation

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ABSTRACT

DESIGN OF A MODE OF DELIVERY FOR MACROBEAD TRANSPLANTATION

by
Jagdip Desai

The macrobead is a form of a biohybrid artificial pancreas (BAP), which is being developed to treat type 1 diabetes [1]. A hypothetical human transplant would require 125 rat equivalent macrobeads. For intraperitoneal transplantation a mode of delivery that allows the placement of several hundred macrobeads into a basket or pouch type device is required.

In this study, four commercially available materials were studied as possible candidates for creating a pouch. These materials are polyvinyl chloride acrylic copolymer (PVC), polyethersulfone (PES), polyvinylidenedifluoride (PVDF), and polytetrafluorethylene (PTFE). Tubular pouches of each material were designed. They then underwent in vivo biocompatibility and in-vitro permeability examinations. Biocompatibility was evaluated by semi-quantitatively analyzing the degree of tissue reaction on the materials surface. Permeability to insulin was quantitatively determined by assaying samples of medium in which pouches containing functioning macrobeads were cultured.

Based on the results two materials PES and PVDF appear suitable for creating a pouch to hold a large number of macrobeads. Additionally, parameters such as pore size and shape of pouch are also critical and require attention.

DESIGN OF A MODE OF DELIVERY FOR MACROBEAD TRANSPLANTATION

by
Jagdip Desai

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DESIGN OF A MODE OF DELIVERY FOR MACROBEAD TRANSPLANTATION

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This thesis is dedicated to my parents, my sisters: Parul, Sunita, and my friends: Raj, Reena.

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CHAPTER 1

INTRODUCTION

Insulin dependent diabetes mellitus (type 1, IDDM), an auto-immune disease occurring in individuals of all ages, affects approximately 1 million people in the United States [2]. These individuals require daily multiple insulin injections [3]. Advances during the last decade increasingly indicate a multifactorial etiology of the disease [4]. The various forms of diabetes and its complications represent the third leading cause of death in the United States. It is estimated that diabetes places a yearly 92 billion dollar strain on America's health care system [5].

1.1 Pathophysiology of Diabetes

In type 1 diabetes, as a result of a cellular/humoral autoimmune attack, the insulin secreting abilities of the beta cells, in the Islets of Langerhans, are destroyed entirely [6-10]. Insulin is the primary hormone which controls the storage and metabolism of ingested foods in the liver, muscle, and fat tissue of the body. The result of insulin deficiency is an increase in blood glucose concentration. This hyperglycemic condition, when maintained for a substantial period of time, directly relates to complications such as kidney failure, blindness, amputation, heart attack, and stroke [8].

1.2 Conventional Treatments

The basic treatment for type 1 diabetes has not changed since insulin became available in the 1920s [8]. It involves the administration of exogenous insulin via injection devices and/or implantable insulin secreting pumps. Attempts have also been made to design aerosolized nasal and oral insulin sprays [11]. Daily injection of insulin requires the burdensome tasks of continuously monitoring blood glucose levels and then calculating the proper dosages for insulin administration. Because this method can be imprecise, the risk of overdosing causing hypoglycemia, or underdosing leading to ketoacidosis is ever-present.

Additionally, although conventional exogenous insulin therapy reduces the incidence of acute diabetic comas, the life expectancy of IDDM individuals continues to remain well below that of the general population [8]. Conventional insulin therapy fails to provide glucose maintenance adequate enough to prevent onset of the long-term complications associated with diabetes [12].

Recent research suggests that many of the complications of diabetes may be avoided or significantly reduced by a more precise and continuous control of blood glucose levels [13,14,15]. Despite intensive work on several exogenous insulin delivery systems, at present, no treatment capable of providing continuous control of blood glucose exists.

1.3 Alternative Treatments

One alternative method for treating type 1 diabetes involves transplantation of whole or segmented portions of the pancreas. This method is attractive physiologically because it will maintain blood glucose in a manner similar to that of a normal individual.

Although pancreatic transplantation would appear to be an ideal method of therapy for insulin dependent diabetics, there are a number of limitations that hinder its large scale utilization. Aside from the initial obstacle of human organ procurement, whole pancreas transplantations are major surgical procedures with high costs. Additionally, transplanted graft survival is dependent upon the administration of life long immunosuppressive drugs. Because immunosuppressive drugs have the potential to cause serious side effects, it usage for protecting whole pancreas transplantations in young "healthy" diabetics would be counterproductive. Currently, most pancreatic transplantations are performed in conjunction with kidney transplantations, which themselves necessitate immunosuppressive therapy [16].

For "healthy" diabetics, transplantations of isolated islets of Langerhans may prove to be the most desirable method of treatment for preventing the onset of long-term complications [17,18]. Islet transplantation requires only minor surgery and thus incurs low risks especially when compared to whole pancreas transplantations.

The transplantation of isolated islets of Langerhans, although promising, is hindered by problems of human donor procurement, isolation and purification techniques, immune rejection, and in-vivo function [17,18,19]. In addition, evidence

of the auto-immune nature of diabetes indicates that unprotected islet transplants will be destroyed by the same sequence of events that destroyed the original pancreas [16].

Auto-immune destruction of transplanted islets might be prevented by purposefully implanting islets whose tissue-typing antigens do not resemble those of the host's original islets [18,20]. Alternatively, many investigators are exploring ways to prevent rejection by encapsulating donor islets into semi-permeable membranes [11,12]. As a result of this latter research, the Biohybrid Artificial Pancreas (BAP) has been developed [18].

1.4 Biohybrid Artificial Pancreas

The BAP consists of insulin secreting islet cells encapsulated by a semi-permeable membrane. The membrane allows the passage of low molecular weight molecules such as glucose, insulin, and nutrients, while it simultaneously protects the islets from rejection by preventing the passage of T-cells or antibody cells of the host immune system [18,20].

At their most general level, BAPs can be categorized as either intravascular or extravascular systems. Although these systems differ greatly in their modes of operation, the basic principle in both remains the same. In BAPs, glucose diffuses through the membrane to the islets, thereby inducing insulin secretion. The insulin, in turn, builds up in the chamber and diffuses through the membrane into the blood supply.

Immuno-isolation systems have several potential advantages over other insulin delivery systems. First, since beta cells in isolated islets of Langerhans maintain their

normal response to glucose and other secretagogues, insulin (and other islet hormones) released from the device can be controlled in a manner similar to a normal individual [21,22,23]. Feedback in blood glucose concentrations as well as responsiveness to changes in the concentrations of other stimuli such as amino acids, fats, and gastric inhibitory polypeptides will help determine the appropriate amount of insulin release. Second, because experimental evidence shows that through isolation and encapsulation techniques, it is possible, without using any immunosuppressive therapy, to prevent rejection of allogenic and xenogenic transplanted islet tissue an unlimited supply of non-human islets become available for transplantations [18,24,25].

The work of several investigative teams has established the validity of the principles upon which immuno-isolation is based. Immune protection via membrane separation has been shown to be a viable concept in general, and with islets in particular [26,27]. Islets have been shown to maintain insulin release during culture in hollow fibers and in microcapsules [26,27,28]. Extravascular devices have remained viable in vivo for greater than 250 days [29,30]. Intravascular shell devices have remained patent for up to 267 days in pancreatomized dogs [29].

In both types of devices, however, continued success is prevented because of complications which arise as a result of their specific designs. Many publications describe how the attachment of an intravascular device to a blood vessel makes the blood more prone to clotting [31]. Discontinuities in blood flow are the major reasons for this clotting. In extravascular devices, the limiting factor arises as a result

of tissue-material interfaces between the device membrane and natural body tissue. Extravascular devices, such as microbeads made from alginate-poly-L -lysine [32], that are transplanted into the peritoneal cavity have a tendency to cause a tissue inflammatory reaction. This reaction results in a layer of fibrous tissue, around the device, ranging from 50 to 100um thick [30]. As a result the diffusion distance from the islets to the blood circulation is extended beyond the point effective for proper metabolic exchange [31].

In addition to tissue inflammatory reactions caused by certain materials, it has been shown that certain geometries of a BAP may also induce the tissue inflammatory cascade. In 1982, Woodward examined the influence of a diffusion chamber's geometry on the occurrence of fibrosis [33]. He showed that a disk-shaped chamber induced a zone of collagen-rich connective tissue around the implant. Other investigations, to assess the potential of tubular devices as BAPs, have been conducted [34]. These studies showed that wider-bore tubular membranes were better able to resist adhesions and fibrous formations [34]. Mitsuo has transplanted tubes 4cm in length and 2mm in inner diameter into the peritoneal cavity of diabetic rats [35]. One month after implantation the tube was examined and found to have extremely thin fibrous tissue deposits on the surface. Moreover, the tube scarcely adhered to surrounding tissue and was easily retrieved.

Altman [36] stresses the importance of eliminating any dead space within the structure, while simultaneously maximizing the diffusion surface areas. Additionally, Ward [37] emphasizes the need for smoothness on the materials outer surface.

1.5 The Macrobead

Recently, the islet purification laboratory of The Rogosin Institute, reported the development of a retrievable, replaceable, biohybrid artificial pancreas, the macrobead, which maintains blood glucose levels in a manner similar to a normal functioning pancreas [1]. In vitro studies showed that five macrobeads, containing 1000 Wistar Furth rat islets, encapsulated in a collagen-agarose matrix, were able to secrete 1.8 to 2.4 units of insulin in 24 hours. Additionally, insulin released from the cultured macrobeads remained constant for at least 154 days. In vivo studies, in which a macrobead was implanted into the peritoneal cavities of chemically induced diabetic B6AF1 mice showed a return to euglycemia within 24 hours. Thereafter, euglycemia was maintained for more than 100 days. Recipient mice had normal responses to glucose tolerance tests, indicating that the islets in the macrobead were functioning as they would in an intact pancreas. The macrobeads' biocompatibility was demonstrated by the absence of any fibrosis or adhesions to peritoneal tissues. Macrobeads, which were removed from the first recipient after 100 days and stored in vitro, were able to maintain euglycemia when retransplanted into other diabetic mice.

These results indicate that collagen-agarose macrobeads (6000-8000μm in diameter) are capable of maintaining long term euglycemia in diabetic recipients. Therefore, macrobeads appear suitable for clinical human xeno-islet transplantations. However, before the macrobeads become the method of choice for treating diabetes, its mode of implantation into the peritoneal cavity requires extensive investigation.

1.6 Mode of Delivery-Scope of Thesis

An average human requires approximately 60 units of insulin per day [30]. Thus a hypothetical human macrobead transplantation would require a minimum of 125 rat equivalent macrobeads (each containing 1000 Wistar Furth rat islets). If these macrobeads are transplanted freely into the peritoneal cavity, there is a danger of their entrapment within an organ. Another complication may occur if the beads break and produce a tissue reaction, resulting in possible adhesions. Finally, when replacement of macrobeads is required, free beads in the peritoneal cavity would be very difficult to retrieve. To overcome these problems, a mode of delivery that allows the placement of several hundred macrobeads into a basket or pouch type device would be desired. Such a device would improve the efficiency of transplantations into larger animals and eventually humans by maintaining all the macrobeads at the site of implantation.

In creating a pouch, compliance with three important criteria is required. First, the material must be permeable to glucose, insulin, and amino acids. Second, the material must be completely biocompatible. Third, the material must have sufficient strength to hold a large number of macrobeads. Immune protection is not a required characteristic because the macrobeads, themselves, are immuno protective.

CHAPTER 2

METHODS

2.1 Commercially Available Materials

Four commercially available synthetic materials have been identified as possible candidates for creating a pouch within the peritoneal cavity. These materials are: 1) Polyvinyl chloride acrylic copolymer, 2) Polyethersulfone, 3) Polyvinylidene-difluoride, and 4) Polytetrafluorethylene (PTFE-Teflon).

Polyvinyl chloride acrylic copolymer (PVC)

The use of acrylic copolymers as a selectively-permeable membrane in a BAP is well documented [30,38,39]. Tubular acrylic copolymer chambers, using xenogenic islet tissues, have been implanted into the peritoneal cavity of spontaneously diabetic rats and pancreactomized diabetic dogs. Graft survival periods range from 60 to 270 days [31]. Upon removal, examination of the BAPs showed very slight fibrosis (from one cell layer to <50um thick).

The Amicon Division of W.R. Grace and Co. manufactures a polyvinyl chloride acrylic copolymer membrane (Amicon XM-50). This membrane is attached to an inert glossy surface which improves its handling capabilities. If this inert coating also increases the membrane's biocompatibility then the Amicon copolymer membrane may be a suitable material for developing a peritoneal pouch.

Polyehtersulfone (PES)

In January 1994, Takagi and Iwata reported a novel microbead using agarose and polystyrene sulfonic acid [40]. The surface of the macrobead was also coated with chondroitin sulfate to enhance the microbead's biocompatibility. When 1,000 hamster islets, encapsulated into microbeads, were transplanted into the peritoneal cavity of STZ-induced diabetic mice, normoglycemia was achieved and maintained for more than 100 days.

Gelman Sciences manufactures polyethersulfone membranes which have high mechanical strength and easy to handle characteristics. Additionally these membranes comply with U.S.P. Class VI-121 plastics tests for biosafety, and have passed cytotoxicity and hemolysis testing. These physiochemical properties of polyehtersulfone indicate that this material, too, may be suitable for encasing large numbers of macrobeads.

Polyvinylidenedifluoride (PVDF)

In 1991, Makino reported a self-regulated insulin delivery system [41]. The device consisted of chemically bound insulin, encapsulated into microcapsules. The microcapsules were then placed into a Durapore polyvinylidenedifluoride membrane pouch. In vitro studies, in which an influx of glucose was administered and an efflux of insulin was measured, showed that this device was able to respond, with no significant lag time, to a glucose challenge.

Millipore manufactures a modified hydrophilic polyvinylidenedifluoride membrane in which the physiochemical properties include: a thickness of 125um, a

porosity of 70% and most importantly, a protein binding ability of only 4ug/cm^2. Additionally, this membrane has a high degree of mechanical strength and so can be folded and pleated without breaking. These properties make the modified polyvinylidenedifluoride membrane an excellent candidate for an intraperitoneal pouch.

Polytetrafluorethylene (PTFE)

Polytetrafluorethylene (Teflon) is the most commonly used material for small diameter artificial vascular grafts [42]. Teflon when transplanted into in vivo systems, is able to maintain its surface integrity without causing any cell adhesions or tissue reactions. Intravascular BAP devices using PTFE grafts in the form of an arteriovenous shunt have been transplanted into diabetic dogs [43]. Device patency has been maintained for 3.5 years while xenogenic graft function was maintained for 106 days [25].

The major concern in using PTFE membranes to design a pouch for peritoneal macrobead transplantation is their hydrophobic characteristics. Most BAP investigators agree that all membranes used in peritoneal transplantations should be hydrophilic [44]. If Teflon's hydrophobicity can be overcome; e.g., by introducing large pores to the membrane surface; while still maintaining it's biocompatible properties, then it may be the material of choice.

Impra, a division of Gore-Tex, manufactures Teflon vascular grafts which come in varying degrees of inner diameter and wall thickness.

2.2 Design of a Delivery System

Designs of a peritoneal pouch, for the sole purpose of maintaining large numbers of immunoprotective micro/macrobeads, have not been described. However, by reviewing the existing literature on proposed micro/macroencapsulated bioartificial pancreas designs, a great deal of information may be obtained [33,34,35,36,37].

Taking this information into account, it would appear that a pouch with a tubular shape would be most desirable. Additionally, although contradicting the requirements of surface smoothness, the efficiency of the macrobead-pouch system may further be enhanced by creating large pores onto the membrane surface. Testing of such a device may show greater responding ability when exposed to a glucose challenge.

2.3 Experimental Procedure

All experiments were performed after the study design and procedures had been examined and approved by The Rogosin Institute's Animal Care and Use Committee. (Protocol 1-1995)

2.3.1 Overview

The experimental design for creating a mode of delivery using commercially available materials was composed of two phases. (Flowchart 2.1). In Phase I the materials underwent in vivo biocompatibility examinations. In Phase II, the materials underwent in vitro permeability examinations. The results of Phases I and II have helped identify any material(s) which may be used to design a mode of delivery for macrobead transplantation.

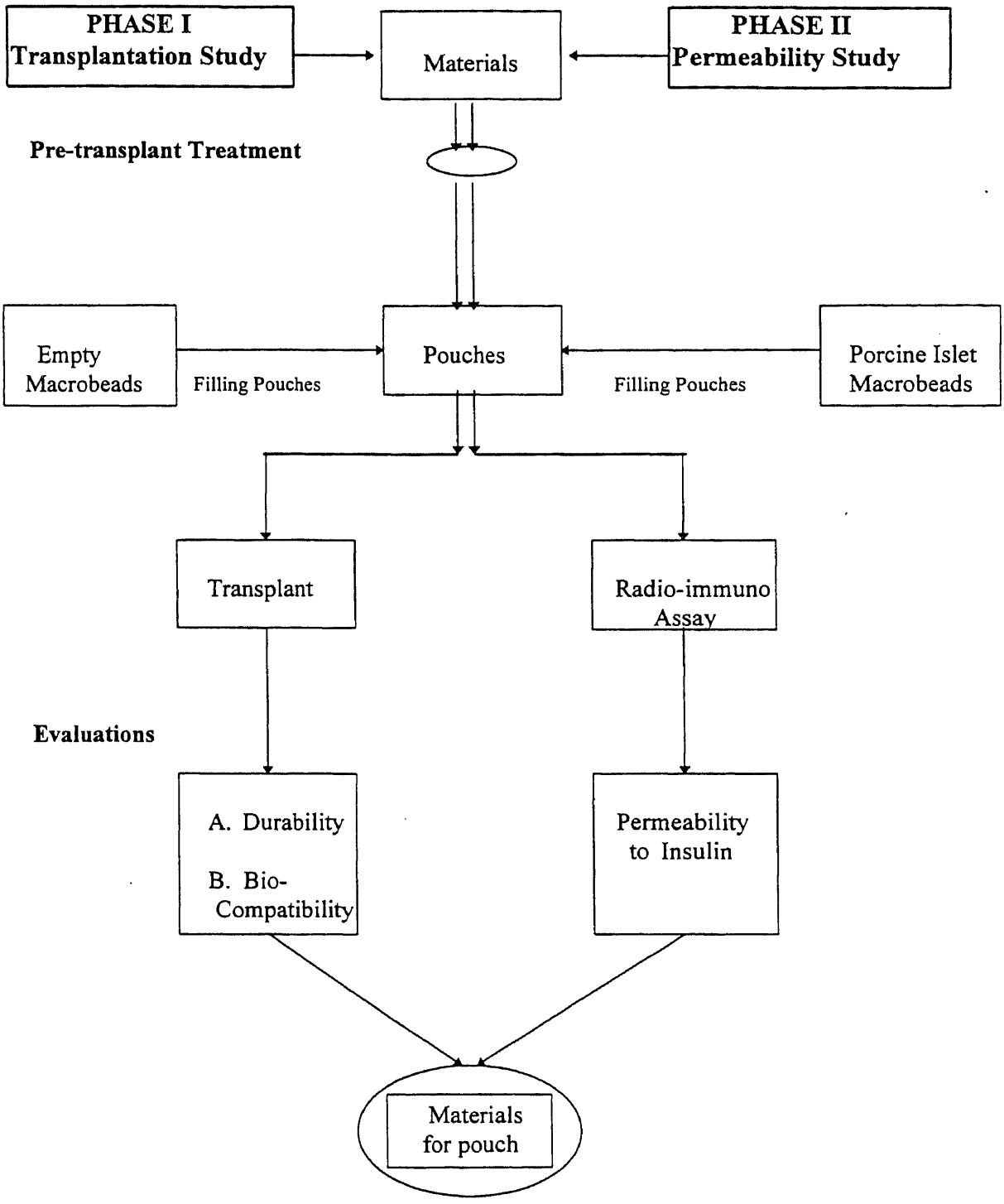


Figure 2.1 Experimental Design

2.3.2 Phase I: In Vivo Transplantations

For in vivo studies, pouches were prepared by manipulating the membranes into tubular shaped devices. In a few pouches (n=3-5), for each material, large pores were introduced onto the membrane surface. This allowed us to determine whether the diffusive transport of molecules can be increased without causing any detrimental effect to the recipient. For each material, 6-10 normoglycemic Wistar Furth rats were transplanted by freely dropping a single pouch into the peritoneal cavity. Transplantation of 6 empty macrobeads into the peritoneal cavity of a single recipient served as a control. To monitor post-surgical recovery, long-term physical condition, and any possible toxic effects due to the materials after intraperitoneal transplantation of pouches, each recipients body weight was determined on the day of surgery and every fourth day after, until explantation of the pouches was performed. At 16 (n=3 for PVDF, PES, and PTFE) and 44 (n=7 for PVDF, PES, and PTFE, n=6 for PVC) days post transplantation the implants were removed. The material's durability (i.e. ability to hold a specific number of macrobeads without rupturing) was evaluated by visually examining all retrieved pouches for physical defects. Each materials biocompatibility (i.e. degree of tissue reaction) was semi-quantitatively evaluated by determining the percent of surface area free of fibrosis.

1. Preparation of pouches

For PVDF, PES, and PVC 10 rectangular sheets (5cm x 3cm) were cut. PTFE comes as a tube with an inner diameter of 19mm. To prepare pouches from PTFE, the tube was cut at 3cm intervals ten times.

For 3 pouches of each material, except PTFE where n=5, large pores (4mm diameter) were created via a stationary hole punching device.

Using a cylindrical tube (10mm diameter) as a mold, PVDF, PES, and PVC membranes were wrapped around into a tubular shape. The 3cm edge was used to wrap around the mold. As a result, 10mm diameter, 5cm long cylindrical pouches were created.

MF cement glue (Millipore, Bedford, MA) was used to seal the materials along the 5cm length of the tube (not required for PTFE). MF cement glue was then added to close one end of the tube.

All the pouches were then sterilized by autoclaving for 20 minutes at 120 °C.

2. Preparation of empty macrobeads [1]

A 5% Agarose solution using Type XII Agarose (Sigma, St. Louis, MO), 1% antibiotic-antimycotic (Gibco, Grand Island, NY) and MEM medium (Gibco) was prepared.

The solution was maintained in a 60 °C water bath.

One hundred microliters of solution was transferred as a single drop into sterile mineral oil at room temperature.

After 1 minute, the now smooth, semisolid macrobead was transferred to RPMI Antibiotic medium (RPMI, 1% antibiotic-antimycotic [Gibco] at 37 °C.

To remove any residual oil, the macrobead was washed 5 times with antibiotic RPMI medium.

The empty macrobeads were stored in an incubator which maintains a condition of 37 °C, with a humidified atmosphere of air and 5% CO_2.

3. Filling pouches with macrobeads

Pouches were soaked in antibiotic RPMI medium at 37 °C for 24 hours.

Using a Teflon coated spatula, 6 macrobeads were placed into each pouch.

The remaining open end was then sealed via MF cement glue.

The macrobead containing pouches were then incubated in complete medium at 37 °C until the day of transplantation.

4. Transplantation of pouches

All surgical tools were sterilized by autoclaving and all surfaces near the transplantation site were wiped thoroughly with 70% ethanol.

Each recipient was anesthetized using a 50mg/kg intraperitoneal injection of pentobarbitol Na, or a combination of 20mg/kg Ketamine and 4mg/kg Xylazine. The ventral side of the animal was shaven to remove outer fur coating. Using an alcohol pad, the ventral surface was wiped to remove any remaining hair fibers. Betadine was then applied, to clean the exposed skin.

Beginning near the bottom of the peritoneal cavity, a mid-line incision through the peritoneal wall was made. The incision was carried for 5cm towards the top of the peritoneal cavity. For the control, only a 2cm cut is required.

The pouches were freely dropped into the peritoneal cavity. For the control, macrobeads were similarly dropped into the peritoneal cavity.

A two layer closure, using an absorbable suture, was performed.

5. Evaluation of pouches

For all transplants, the animal's body weights were measured on the day of surgery and every fourth day after until explantation of the pouches was performed.

At the end of the desired incubation periods (16 days and 44 days) all animals were sacrificed.

Implants and surrounding tissues were retrieved and then fixed in 4% buffered formalin.

Each membrane pouch and the beads within were visually examined for physical defects (e.g. tears).

For assessing biocompatibility, a semi-quantitative method for determining the percent of surface area which is free of any fibrosis was used. To simplify calculations, all pouches were flattened into a rectangular shape. Total surface area of the pouch was then calculated by the formula: (2 x length x width). Surface area which is free of fibrosis was then calculated. The areas free of fibrosis were simplified into rectangular shapes. The area of the rectangles were calculated by multiplying length x width. For the pouches with large pores, the area of each pore was taken into account by first determining whether the pore is open or closed. If a pore was open, then its surface area was added to the amount of surface area free of fibrosis. The area of each pore was calculated by using the formula for determining the area of a circle (πr^2 where r equals 2mm, the radius of the pores). The percent of surface area free of fibrosis was then calculated by the formula:

$$\%\text{Surface area free of fibrosis} = \frac{\text{surface area free of fibrosis}}{\text{total surface area}} \times 100$$

2.3.3 Phase II: In Vitro Permeability

In Phase II each material's permeability to insulin was quantitatively determined by assaying samples of medium in which pouches containing functioning macrobeads were cultured. Insulin release in a 24hr time period was calculated for 12 samples of macrobeads (3 for each material). The macrobeads of each sample were then placed into pouches and assayed again to determine the amount of insulin release.

1. Preparation of pouches

   The same procedure as described in Section 2.3.2 Step 1, was used, except no large pores were introduced onto the pouch surface.

2. Procurement of functioning macrobeads

   Porcine insulin secreting macrobeads were obtained from The Rogosin Institute's Islet Purification Laboratory.

3. Assessing insulin release from macrobeads

   Twelve dishes each containing a specific number of porcine insulin secreting macrobeads were cultured in 50ml of RPMI medium with 25mM HEPES [Gibco, Grand Island, NY] 10% heat-inactivated porcine serum [Cellgro, VA], and antibiotic-antimycotic solution

1ml/100ml [Gibco] (hereinafter referred to as complete medium) in a humidified atmosphere of 5% CO_2 and 37 °C. The medium in the dishes was changed once a week.

To determine the amount of insulin release in a 24 hour time period, the medium in each dish was changed an additional time. After 24 hours, a 2ml sample of medium was collected from each dish and immediately frozen and stored at -20 °C until radioimmunoassay was performed.

Insulin release in the medium was determined by radioimmunoassay (RIA) using a kit for measuring porcine insulin [Linco Research Inc., St. Charles MO]. (Appendix A).

4. Encasing functioning macrobeads into a pouch

All pouches were soaked in antibiotic RPMI medium at 37 °C for 24 hours.

To eliminate the possibility of any dead space (e.g. air bubbles) existing within the pouch, each pouch was completely filled with 0.5% agarose solution. Macrobeads were then placed inside the pouch and suspended in the 0.5% agarose solution.

Using a Teflon spatula, all the macrobeads in a dish were placed into a pouch containing 0.5% agarose solution. As each bead was dropped, an equivalent volume of 0.5% agarose was displaced.

After placement of the final macrobead, the open end of the pouch was plugged using 5% agarose.

Once the agarose plug solidified, the pouch was incubated in 80ml (100ml for PTFE) of complete medium.

For each of the four materials, three pouches were filled with functioning macrobeads and assayed for insulin release.

5. Evaluation of insulin release from the pouches

To determine the amount of insulin release in a 24 hour time period, the medium in each dish was changed. After 24 hours, a 2ml sample of medium was collected from each dish and immediately frozen and stored at -20 C° until radioummunoassay was performed. This procedure was performed on 1, 7, and 16 days post-encasement.

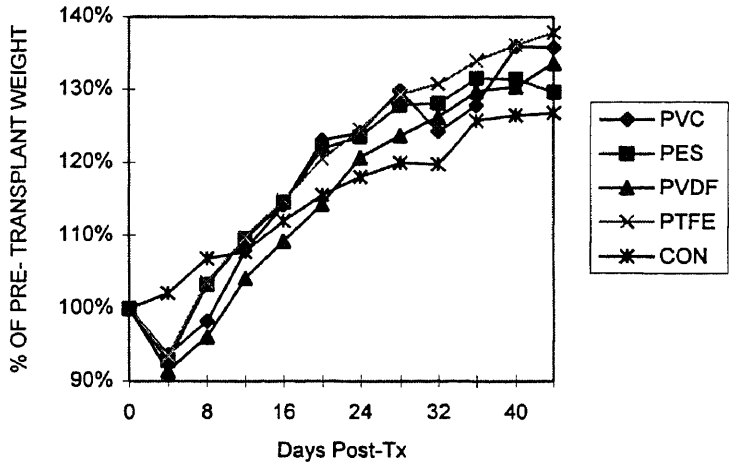
Insulin release in the medium was determined by RIA.

CHAPTER 3

RESULTS

3.1 Clinical Evaluations after Transplantation

Figure 3.1 shows the mean post-surgical weight gain of animals over a period of 44 days for transplants of each material. Individual data for each transplanted animal is listed in Appendix C.



Mean weight gain of transplanted animals for transplants of each material. Results are expressed as the percentage of the weight measured on the day of surgery (day 0). Sample size: PVC (n=6), PES(n=4), PVDF(n=7), PTFE(n=6), CON(n=1).

Figure 3.1 Post Surgical Weight Recovery

Except for two recipients, the control and an animal receiving a PVC membrane pouch, all transplanted animals experienced weight loss at 4 days post surgery. Transplants with PVDF membrane pouches experienced an average

weight loss of 16.8±2.7 (±SEM) grams. Similarly, recipients of PTFE, PVC, and PES membrane pouches experienced average weight losses of 18.8±4.2, 19.0±8.4, and 23.4±3.25 grams, respectively. At four days post surgery the control (i.e. intraperitoneal transplantation of 6 empty macrobeads) had full recovery and in fact had gained 5.1 grams. The transplants who received macrobeads enclosed in pouches showed delayed recovery. By the 12th day of recovery, 21 of 23 transplanted animals regained their pre-surgery body weight. They then continued to gain weight until 44 days post-surgery, at which point the pouches were removed for evaluation.

3.2 Effects of 4mm Pore Size

Table 3.1 compares the results obtained from implanting pouches with large (4mm) pores to the results obtained from implanting pouches which did not have large pores. Of 14 transplants with large pores, 6 recipients died within 8 days of implantation. In tranplants without large pores all animals survived (24/24). With pouches having large pores intestinal obstruction was observed and was probably the cause of death in all six cases. The 4mm pores allowed the intestines to enter and entangle within the pouch. Figure 3.2 visually describes this phenomena.

Table 3.1 Pouches with large (4mm) pores vs. Pouches without large pores

Type of Pouch:	With 4mm pores	Without pores
Total No. of Transplants	14	24
Total No. of Deaths	6	0
% Mortality	42.9	0

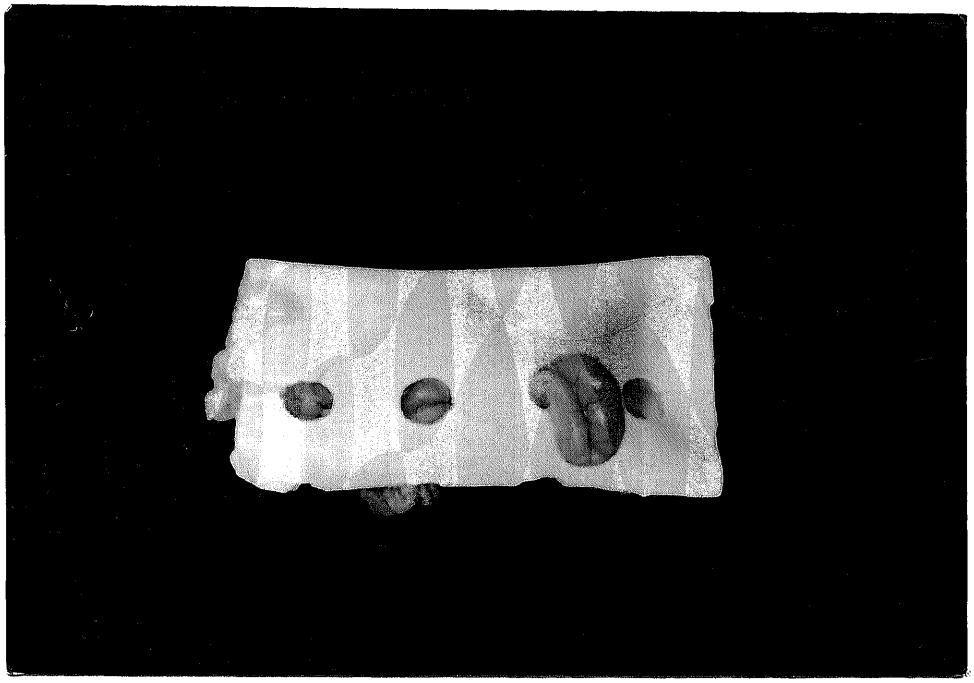
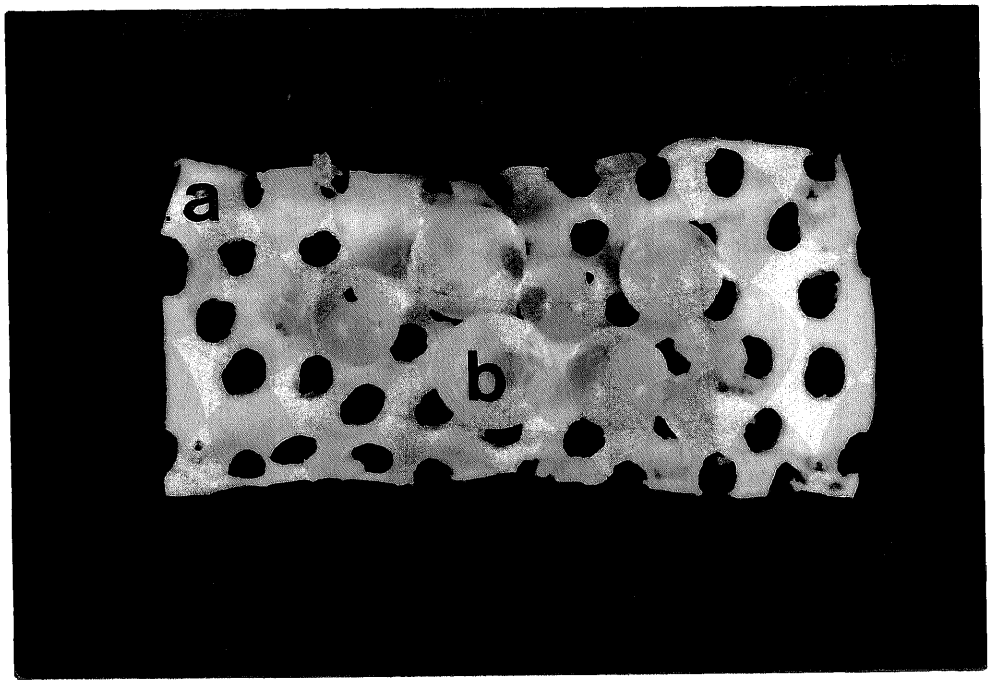


Figure 3.2 Intestinal obstruction in pouches with large pores (10X)

3.3 Durability of Pouches

Examination of the retrieved pouches indicate that all four materials are capable of withstanding the forces encountered in the peritoneal cavity. There were no tears or physical defects in any of the pouches. However, in 6 of the 38 transplanted pouches (n=1 for PES and PVDF, n=4 for PVC) a slight opening of the ends was observed. Examination of the encased macrobeads revealed that in 4 of the 38 transplants (n=2 for PVC and PVDF), broken macrobeads were present. In all remaining pouches the macrobeads remained intact. Figure 3.3 shows an opened pouch, containing intact macrobeads.

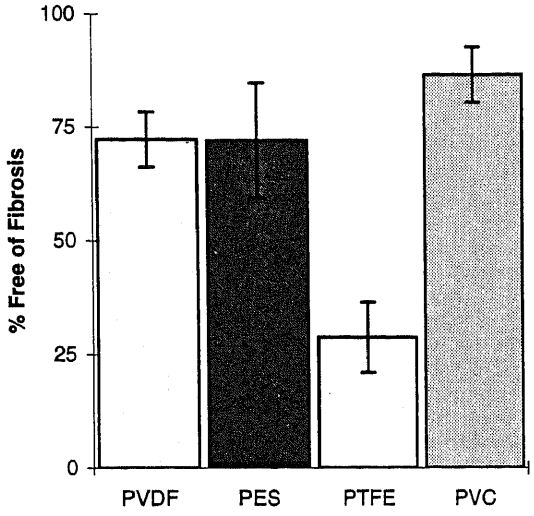


A- PTFE pouch with large pores
B- Empty Macrobeads

Figure 3.3 Opened PTFE pouch with empty macrobeads (10X)

3.4 Biocompatibility of Materials

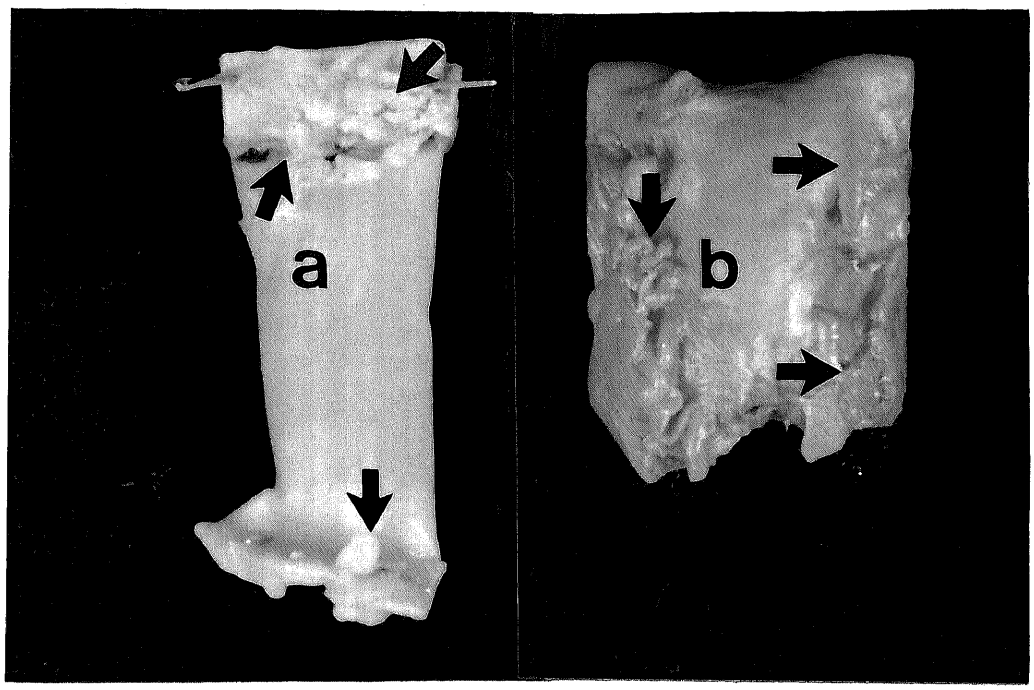
Semi-quantitative analysis of the tissue reaction, as documented in figure 3.4, shows that after 44 days implantation freely dropped PVC membrane pouches had 86.5±6.1% of its surface area free of fibrosis. PVDF and PES pouches had 72.3±6.0 and 72.0±12.7 percent surface area free of fibrosis. Teflon, with 28.7±7.7%, had a smaller amount of surface area free of fibrosis. Individual data, for each pouch material, including those in which pouches were removed on the 16th day post-implantation or earlier because of death, is listed in Appendix C.



Tissue reaction, expressed as the percent of surface area free of fibrosis, for pouches of each material. PVC (n=6), PVDF (n=7), PES (n=4), PTFE (n=6).

Figure 3.4 Percent of Surface Area Free of Fibrosis After 44 days Implant.

In PVDF, PVC, and PES the majority of fibrosis occurred at the ends of the pouches. Teflon pouches, on the other hand, experienced fibrous formations which were evenly spread throughout the total surface area. In one PES and two PTFE pouches adhesion to the liver occurred. Figure 3.5 shows the tissue reactions in a PES and a PTFE pouch respectively.



A- PES pouch B- PTFE pouch
⟶- Fibrous Tissue

Figure 3.5 Tissue Reactions of PES and PTFE Pouches (10X)

3.5 Permeability to Insulin

Table 3.2 summarizes the results of insulin release on 1, 7, and 16 days post-encasement from macrobeads within membrane pouches. Macrobeads encased in PVC membranes secreted 21.1±6.7 (±SEM), 19.0±4.4, and 11.8±3.1 percent of their pre-encasement values on 1,7, and 16 days post encasement. Insulin release from the pouches created with PTFE had mean percents of 20.1±3.9, 38.4±9.6, 28.3±1.9 while PVDF had mean percentages of 95.5±8.9, 83.3±8.1, and 79.2±3.9. For PES the mean percentages were 107.2±9.8, 106.2±3.3, and 77.2±1.9 on days

macrobeads was measured on the same days as those which were placed in a membrane, had 90.4, 91.5, and 104.8 percent of its initial insulin release.

Table 3.2 Insulin release from Macrobeads before and after Encasement into Membrane Pouch

Material	Insulin Release of Macrobeads before Encasement (mu/dish24hr)	Insulin Release of Macrobeads after Encasement (mu/dish-24hr) Days Post- Encasement		
		1	7	16
PVC	53.9	9.4	8.4	< 5
PVC	48.3	15.9	13.4	8.7
PVC	123.8	15.9	16.9	10.2
PVDF	75.9	79.1	74.8	58.1
PVDF	27.4	28.5	22.0	23.8
PVDF	59.7	46.8	42.4	44.2
PTFE	100.1	16.3	29.0	25.9
PTFE	120.6	19.4	34.6	32.4
PTFE	53.3	14.8	30.7	17.2
PES	183.6	228.9	190.8	145.7
PES	252.1	267.9	284.2	198.9
PES	166.2	150.9	169.2	121.9
Control (no encasement)	64.6	58.4	59.1	67.7

CHAPTER 4

DISCUSSION

4.1 Clinical Evaluations after Transplantation

The animal weight data, by showing complete recovery, indicates that all of the tested materials are non-toxic and, hence, may be considered biomaterials. The weight loss experienced by recipients of membrane pouches may be attributable to the larger (5cm), more traumatic incision required to insert the pouches. To insert macrobeads alone, only a 2cm incision was required. Additionally, because the pouches are larger than individual macrobeads, subject animals may have initially felt a greater degree of discomfort upon implantation of a pouch. This discomfort could have magnified weight loss by affecting the animals' moods and appetites.

4.2 Effects of 4mm Pore size

The effects observed from transplanting pouches suggests that potentially serious complications may arise from dropping large pore pouches freely into the peritoneal cavity. As designed for this study, the tubular pouches were rigid, nonflexible devices. Upon intra-abdominal pressures, these devices allowed penetration of intestinal tissue through the 4mm pores. In rats, therefore, large pore pouches are not suitable. A possible solution may be to reduce the pore size.

4.3 Durability of Pouches

The strength of the membrane pouches is an important factor in designing a mode of delivery for macrobead transplantation. Within the peritoneal cavity the pouches will encounter both direct and shearing forces. Examinations of the retrieved pouches indicate that all four materials have strength sufficient enough to allow for intraperitoneal implantation. The opening at the ends of several pouches suggests that either an inadequate amount of glue was added or that the Milipore MF cement glue was not effective in properly sealing the end of the pouch. Alternative methods of closure, e.g. stitching or stapling, should be investigated. To prevent crushing and breaking of macrobeads, when encased in a semi-tubular pouch, it may be necessary to suspend the beads in an agarose gel matrix. The matrix, in addition to providing support, may increase the efficiency of the mode of delivery by preventing dead space, e.g. air bubbles from forming adjacent to the macrobeads.

4.4 Biocompatibility of Materials

Analysis of tissue reaction suggests that PVC, PVDF, and PES membranes may be suitable materials for intra-peritoneal transplantation. Fibrosis at the ends of these pouches was minimal and hence should not affect the functioning abilities of encased macrobeads. The adhesion of freely dropped pouches to the liver, however, is a serious concern that must be addressed. To minimize the possibility of adhesions and abscess formations Lanza has suggested localizing devices in a specific place [38]. A pouch for example, could be immobilized against the peritoneal wall.

4.5 Permeability to Insulin

The insulin release data shows that PVDF and PES membranes are more permeable to insulin than PVC and PTFE membranes. The data obtained for PVC membranes is surprising because this material has been used by researchers for islet transplantation [38,39]. Pores, for the PVC membranes used in this study, had a molecular weight cutoff of 50,000 daltons. Insulin has a molecular weight of 6,000 daltons, and so should easily pass through. Through communications with Amicon Inc., the manufacturers of PVC membranes, a probable explanation for our results was derived. PVC membrane filters, unlike the PVC hollow fibers used by other investigators, have an inert glossy coating on one side. This coating, described as the 'skin' of the membrane, allows for one directional flow. In our PVC pouches, the skin remained on the outside, i.e. the side exposed to the medium. Hence, glucose and nutrients in the medium were able to enter the pouch and nourish the islets. However, because of the one way flow, very little insulin produced by the islets was able to cross the membrane and enter into the medium.

The reduction of insulin release from macrobeads when encased in PTFE pouches can most likely be attributed to the membrane's hydrophobicity. Transport of glucose and other nutrients into the pouch, as well as diffusion of insulin out of the pouches depends upon the hydrophilic properties of the material. Hydrophilic membranes, by absorbing the medium, would be more conducive to insulin and glucose transport.

4.6 Phase I & II Summary

In Phases I & II, pouches of each material underwent design, durability, biocompatibility, and permeability examinations. The results have provided insights for designing a mode of delivery for macrobead transplantation.

By comparing the materials to each of the above parameters, PVDF and PES membranes seem to be superior for intraperitoneal transplantation. Their qualities of durability and biocompatibility indicate that they would be able to remain implanted in the peritoneal cavity for an extended period of time. Permeability tests show that when exposed to glucose, macrobeads encased in either of these two membranes will be able to respond.

CHAPTER 5

CONCLUSIONS

5.1 Materials for Pouches

In designing a mode of delivery for macrobead transplantation, four materials were tested. Based upon the results, two materials: Polyethersulfone (PES) and Polyvinylidenedifluoride (PVDF) appear suitable for encasing a large number of macrobeads. If the permeability of PVC membrane filters can be modified to allow unhindered flow in both directions, then it also may be suitable for macrobead encasement.

It is important to note here, that only four materials were tested. Investigations of other commercially available biomaterials may find a more promising candidate. Polyvinyl alcohol and Polyacrylonitrile are two additional materials which have been cited in the literature for biocompatibility and selective permeability [35,45,46]. In 1992, Mahgoub reported the ability to achieve euglycemia in diabetic Wistar Furth rats by transplanting xenogenic islets which were encased in rat amniotic membranes [47]. If similar results using xenogenic amniotic membranes can be demonstrated, then placement of macrobeads into such membranes may also be a possibility.

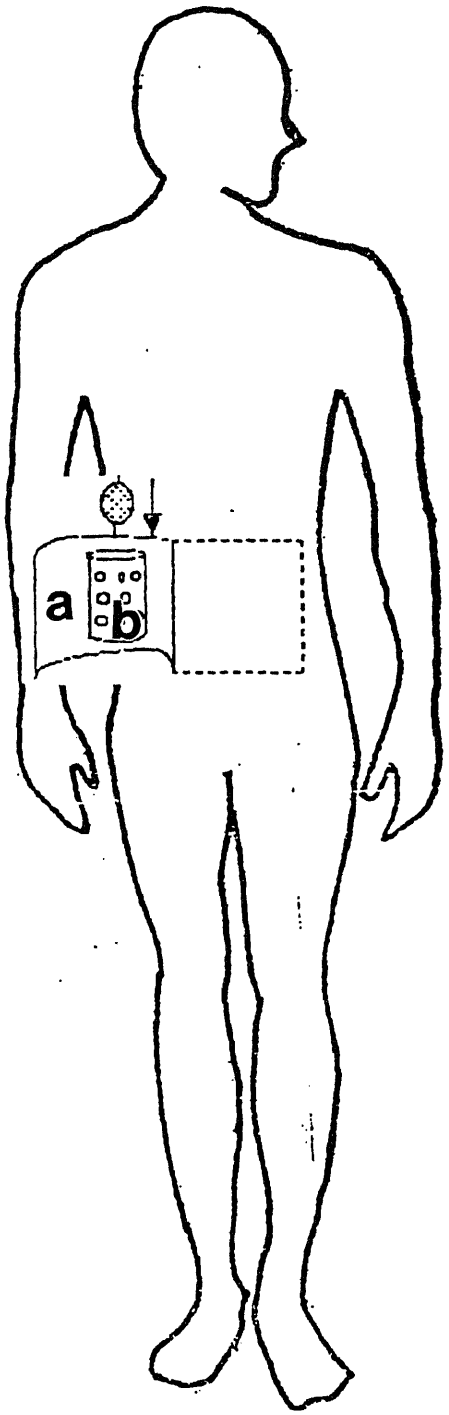
Physiochemical treatments, which modify a specific property, is an additional method which can be used to improve the efficacy of existing membranes. For example, the electrical discharges of the Corona Surface Treatment has been shown to

improve the insulin permeability of acrylonitrile membranes [48]. Utilization of such physiochemical treatments may allow the consideration of what otherwise would be non-useful materials.

5.2 Pouch Design

The pouches designed for this study were rigid semi-tubular devices. PES, PVC and PVDF pouches experienced fibrosis at the ends where the surface was rough and tactile. Clearly, to avoid such tissue reactions, the device must be smooth throughout the entire surface area. Redesigning the pouch either so it is more flexible, or so it can be immobilized against the peritoneal wall may prove effective in preventing fibrotic and adhesive formations. A flexible pouch, like a fishing net which would yield and change shape when exposed to pressure, could be created by crosslinking a pliable fiber material into a type of crochet mesh [49]. Thin PES hollow fibers are commercially available.

Figure 5.1 shows a hypothetical design for immobilizing a macrobead-pouch graft against the peritoneal wall. Through immobilization, contact between the pouch and peritoneal organs can be limited.



a- Peritoneal Wall b- Membrane Pouch

⟶ - Insertion of Macrobead into Membrane Pouch

Figure 5.1 Immobilizing a pouch against the Peritoneal Wall

5.3 Future Experiments

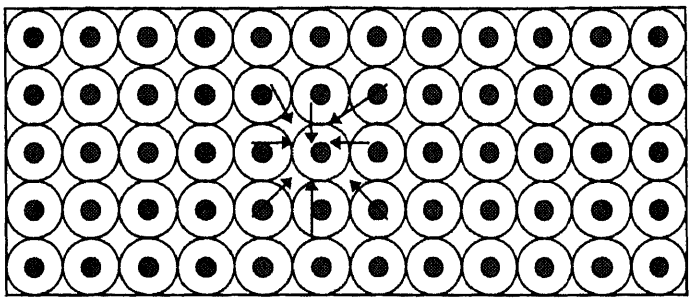
In this study, potential materials and preliminary methods to evaluate these materials for macrobead transplantation have been described. The next step is to design protocols which, in greater detail, study a pouche's biocompatibility and permeability.

In assessing biocompatibility, we semi-quantitatively determined the degree of tissue reaction on the pouche's outer surface. This gave us a general description on the material's compatibility. However, in order to thoroughly evaluate a material, an understanding of the reactions occurring at the tissue-material interface should be obtained. Histological examinations of retrieved pouches could tell us what is happening on the membrane's surface. It would be interesting to see whether the membrane's pores remain open or become clogged after intraperitoneal transplantation. Experiments in which sections of retrieved pouches are subjected to histological examinations should be conducted before any conclusive assessment on the material is made.

To determine the permeability to insulin, 6-10 porcine macrobeads were inserted, in single-file fashion, into pouches of each material. The results showed that for two materials, there was no reduction in insulin secretion from the macrobeads. For transplantation into large animals a greater number of macrobeads will be required. As a result, placement of macrobeads in single-file fashion may not be possible. A pouch which maintains macrobeads in multiple, adjacent columns may be required. However, because transport into and out of the macrobeads is based on diffusion with the surrounding environment, the beads located in the center of such a

design may not be able to function as they normally would. The ability of these beads to assess glucose concentrations and then respond with insulin secretions might be affected by the functioning of adjacent macrobeads. Figure 5.2 graphically explains this potential phenomena. In vitro experiments in which a hypothetical transplantation number of macrobeads are placed into a pouch and then assayed for insulin release should be performed. Additionally to assess their response abilities, these pouches should be challenged with varying concentrations of glucose.

Ultimately, the efficacy of a mode of delivery for macrobeads will be determined by performing transplantations into diabetic recipients. Experiments in which macrobead-pouch grafts are compared to controls of unencased macrobeads, non-transplanted diabetic animals, and non-transplanted normal recipients should first be performed. Body weight, blood glucose levels, blood c-peptide levels, and response to glucose tolerance tests are parameters to be measured. To assess whether immobilization of a device is effective in preventing adhesions, transplantations into large animal models (e.g. rabbits, cats, and dogs) should be performed.



⟶ direction of insulin release from adjacent macrobeads

The macrobead, in the center of the pouch might, because of secretion from adjacent macrobeads, sense high insulin levels. Hence it will not secrete any insulin. As a result, the net insulin release from the pouch may be less than the sum of insulin release from the individual macrobeads.

Figure 5.2 Placement of Macrobeads into pouch

APPENDIX

A- PORCINE INSULIN RIA

B- INDIVIDUAL ANIMAL WEIGHT DATA

C- PERCENT FIBROSIS FREE DATA

PORCINE INSULIN RIA KIT

250 TUBES

Reagents Supplied

This insulin assay has been developed for the measurement of porcine insulin in plasma or serum. The tracer is prepared with human insulin. All reagents are ready for use. Refrigerate upon arrival.

1. Assay buffer (0.05M Phosphosaline pH 7.4 containing 0.025M EDTA, 0.01% EMTSA and 1% BSA), 40 ml.
2. Insulin Antibody, 26 ml.
3. ^{125}I-Insulin Label. Hydrate using entire contents (27 ml) of "label hydrating buffer."
4. Label Hydrating Buffer (containing normal guinea pig IgG as a carrier). 125I-Insulin must be hydrated with entire contents of label hydrating buffer.
5. Human Insulin Standards, 2 ml each
   2, 5, 10, 20, 50, 100, 200 µU/ml
6. Quality Controls I (low) and II (high), 2 ml each
7. Precipitating Reagent, 260 ml.

Procedure

Use Borosilicate glass tubes (12 x 75 mm) for assay procedure.

Assay Set-up

1. Pipette 300 µl of assay buffer to the non-specific binding (NSB) tubes (3-4) and 200 µl of buffer to reference (Bo) tubes (5-6) and add 100 µl of buffer to tube 7 to end of assay.
2. Pipette 100 µl of standards and quality controls in duplicate. (see Contents of Tubes).
3. Pipet 100 µl of sample in duplicate. (NOTE: when smaller volumes of sample are used, additional buffer should be added to compensate for difference so that volume is equivalent to 100 µl, e.g. when using 50 µl sample, add 50 µl buffer).
4. Pipette 100 µl of ^{125}I- insulin to all tubes.
5. Pipette 100 µl of porcine antibody to all tubes except totals (1-2) and NSB (3-4).
6. Vortex, cover and incubate overnight (18-24 hours) at *4°C.*

* a value is to high if its cpm is lower than S7

* a value is to low if it cpm is higher than S1.

Next Morning

7. Add 1.0 ml of precipitating reagent to all tubes (except totals).
8. Vortex and incubate 20 minutes at 4° C.
9. Centrifuge for 15 minutes at 2,000-3,000 x g. 3700 26 minutes / NO Brake
10. Decant supernatant, drain tubes for 1 minute, and count pellet.

Contents of Tubes/Assay Procedure Chart

Tube Number	Buffer	Standard/QC Sample	Label	Insulin Antibody	Precipitating Reagent
(T) 1,2	-	-	100 µl	-	-
N 3,4	300 µl	-		-	1.0 ml
So 5,6	200 µl	-		100 µl So	
7,8	100 µl	100 µl of 2 µU/ml (S1)			
9,10		5 µU/ml (S1)			
11,12		10 µU/ml (S1)			
13,14		20 µU/ml (S1)			
15,16		50 µU/ml (S7)			
17,18		100 µU/ml (S7)			
19,20		200 µU/ml (S7)			
21,22		100 µl of QC I			
23,24		100 µl of QC II			
25-n		100 µl of unknown			

Vortex, Incubate 18-24 hrs. at 4° C.

3:1 Tubes

Unknown Dilutions

1:51 -

20 ul sample + 1000 ul Buffer = 1020 ul

$\frac{20}{1020} = \frac{1}{51}$

Calculations

The calculations for insulin can be automatically performed by a gamma counter with data reduction system. The log of the known concentration (standards) is plotted as X versus the logit B/REF [unknown bound counts/zero standard bound counts (B/Bo)] plotted as Y. The log/logit function is used to linearize the curve by five cycle weighted regression analysis. Results are reported as µU/ml insulin of unknown sample. [NOTE: When sample volumes assayed differ from 100 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g. if 50 µl of sample is used, then calculated data must be multiplied by 2)].

Manual Calculation

1. Average duplicate counts for NSB (tubes 1,2), Total Counts (tubes 3,4) and Total Binding (tubes 5,6), and remaining samples.
2. Subtract the average NSB counts from each average count (except for Total Counts). These are the counts used in the following calculations.
3. Calculate the percentage of tracer bound [(Total Binding Counts/Total Counts) x 100]. This should be 35-40%.
4. Calculate the percentage of maximum binding (%B/BO) for each standard and sample [%B/BO = (Sample or Standard/Total Binding) x 100].
5. Plot the % B/BO for each standard on the y-axis and the known concentration of the standard on the x-axis. The use of log-log graph paper will result in a nearly linear curve.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the insulin of the unknown samples (unknowns and controls) by interpolation of the reference curve.

Limits of Test Procedure

1. Assay should be rejected if one of the two QCs falls outside of 2 SDs.
   See the supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.
3. The limit of sensitivity for the insulin assay is 2 µU/ml (100 µl sample size).
4. The limit of linearity for the insulin assay is 200 µU/ml (100 µl sample size). Any result greater than 200 µU/ml should be repeated on dilution using assay buffer as a diluent.

Interpretation

Normal Fasting Range: 5-15 µU/ml

Performance

ED_{80} = 7 ± 1 µU/ml

ED_{50} = 29 ± 2 µU/ml

ED_{20} = 122 ± 11 µU/ml

Crossreactivity

Porcine Insulin	100%
Human Insulin	100%
Human Proinsulin	38%
Des 31,32	47%
Des 64, 65	72%
Glucagon	Not Detectable
Somatostatin	Not Detectable
Pancreatic Polypeptide	Not Detectable

References

1. Morgan, CR and Lazarow, A. Immunoassay of insulin: Two antibody system. Plasma insulin levels in normal and diabetic rats. Diabetes 12: 115, 1963.

Reference date: 10/15/92

Replacement Reagents	Cat. #
1. Assay Buffer	AB-P
2. Porcine Insulin Antibody	1012-K
3. 125I-Insulin, with label hydrating buffer	9011-K
4. Human Insulin Standards	8012-K
5. Quality Controls	6000
6. Precipitating Reagent	PR-UV

Signature Sharon Meyer

Appendix B INDIVIDUAL ANIMAL WEIGHT DATA

PVC

Animal	Day Post Tx	weight	% of zero wt.	Animal	Day Post Tx	weight	% of zero wt.
23-1R	0	204.2	100%	42-1R	0	275.4	100%
	4	191.1	94%		4	247.7	90%
	8	200.6	98%		8	261.3	95%
	12	220.4	108%		12	276.5	100%
	16	233.1	114%		16	285.2	104%
	20	251.4	123%		20	291.2	106%
	24	253.4	124%		24	300.2	109%
	28	265.1	130%		28	305.3	111%
	32	253.8	124%		32	312	113%
	36	261	128%		36	316.2	115%
	40	277.5	136%		40	320.8	116%
	44	277.4	136%		44	320.3	116%
29-1R	0	252.7	100%	33-1L	0	230.3	100%
	4	231.5	92%		4	255.6	111%
	8	248.2	98%		8	269.1	117%
	12	263.5	104%		12	283.6	123%
	16	277.6	110%		16	290.2	126%
	20	288.2	114%		20	299.3	130%
	24	299.8	119%		24	299.6	130%
	28	303.4	120%		28	306.1	133%
	32	307.3	122%		32	311	135%
	36	311.1	123%		36	317.7	138%
	40	316.2	125%		40	320.9	139%
	44	317.5	126%		44	325.6	141%
33-1R	0	275.7	100%	42-0	0	293.3	100%
	4	246.7	89%		4	266.1	91%
	8	268.8	97%		8	283.6	97%
	12	281.6	102%		12	295.1	101%
	16	289.5	105%		16	305.7	104%
	20	295	107%		20	315.2	107%
	24	301.6	109%		24	320.6	109%
	28	303.3	110%		28	329.3	112%
	32	309	112%		32	331	113%
	36	312.2	113%		36	334.6	114%
	40	318.2	115%		40	339.2	116%
	44	322.7	117%		44	343.2	117%
24-1L	0	193.8		24-1R	0	199.6	
	4	153.4			4	DIED	
	8	DIED					

Animal	Day Post Tx	weight	% of zero wt.	Animal	Day Post Tx	weight	% of zero wt.
41-0	0	207.5	100%	41-1R	0	216.4	100%
	4	185.3	89%		4	196.1	91%
	8	215.5	104%		8	232.1	107%
	12	228.9	110%		12	241.3	112%
	16	238.3	115%		16	255	118%
	20	254.3	123%		20	280.3	130%
	24	256	123%		24	282.5	131%
	28	264.8	128%		28	298.3	138%
	32	264.8	128%		32	291.9	135%
	36	270.3	130%		36	301.2	139%
	40	274.8	132%		40	305.4	141%
	44	282.6	136%		44	310.8	144%
26-1L	0	200.8	100%	27-0	0	237.2	100%
	4	193	96%		4	224.8	95%
	8	211.7	105%		8	238.2	100%
	12	224.3	112%		12	247.6	104%
	16	232.7	116%		16	259.1	109%
	20	244.2	122%		20	263.3	111%
	24	255.6	127%		24	268.4	113%
	28	265.6	132%		28	284.3	120%
	32	275.6	137%		32	285.3	120%
	36	280.5	140%		36	283.9	120%
	40	286.1	142%		40	295.9	125%
	44	287.6	143%		44	292.4	123%
30-1L	0	207.6	100%	26-1R	0	212.7	100%
	4	195	94%		4	203.6	96%
	8	212.5	102%		8	218.3	103%
	12	229.3	110%		12	234.1	110%
	16	245.5	118%		16	239.6	113%
	20	255.7	123%		20	245.1	115%
	24	272.3	131%		24	258.5	122%
	28	277.1	133%		28	265.3	125%
	32	283.2	136%		32	273.1	128%
	36	298.9	144%		36	278.6	131%
	40	298.8	144%		40	280.9	132%
	44	306.7	148%		44	283.1	133%
39-1L	0	204.5		39-0	0	215.9	
	4	191.4			4	195	
	8	222.6			8	228.6	
	12	234.6			12	241.9	
	16	243.5			16	239.5	
39-1R	0	222.8		41-1L	0	198.2	
	4	206.6			4	144	
	8	237.4				DIED	
	12	251.3					
	16	261.9					

Animal	Day Post Tx	weight	% of zero wt.	Animal	Day Post Tx	weight	% of zero wt.
35-0	0	206.7	100%	34-0	0	195.2	100%
	4	192.7	93%		4	176.9	91%
	8	203.5	98%		8	191.1	98%
	12	221.8	107%		12	214.9	110%
	16	236.6	114%		16	221.1	113%
	20	248.3	120%		20	237.2	122%
	24	263.7	128%		24	252.6	129%
	28	266.1	129%		28	257.4	132%
	32	269.4	130%		32	258.6	132%
	36	275.5	133%		36	264.2	135%
	40	273.6	132%		40	260.3	133%
	44	284.2	137%		44	271.4	139%
34-1R	0	195.5	100%	28-1R	0	226.4	100%
	4	184.9	95%		4	215.1	95%
	8	191.3	98%		8	228.8	101%
	12	211.4	108%		12	240.8	106%
	16	217.8	111%		16	256.1	113%
	20	228.1	117%		20	257.7	114%
	24	241.6	124%		24	261.8	116%
	28	248.9	127%		28	269.9	119%
	32	250.7	128%		32	270.4	119%
	36	259.4	133%		36	271.6	120%
	40	257.9	132%		40	275.9	122%
	44	268.8	137%		44	274.5	121%
22-2R	0	210.5	100%	22-1L	0	198.3	100%
	4	173.1	82%		4	174.9	88%
	8	179.6	85%		8	175.1	88%
	12	185.3	88%		12	197.7	100%
	16	187.3	89%		16	213.3	108%
	20	196.3	93%		20	229.7	116%
	24	216	103%		24	237.2	120%
	28	224.4	107%		28	244.9	123%
	32	239.9	114%		32	252.9	128%
	36	249.7	119%		36	263.9	133%
	40	256.2	122%		40	265.5	134%
	44	263	125%		44	269.2	136%
27-1R	0	201.6	100%	37-1R	0	202.9	
	4	193.1	96%		4	190.7	
	8	209.6	104%		8	202.3	
	12	220.5	109%		12	221.7	
	16	233.8	116%		16	234.2	
	20	240.3	119%	37-0	0	202.7	
	24	255.2	127%		4	185.9	
	28	259.6	129%		8	199.6	
	32	266.2	132%		12	223.3	
	36	272.6	135%		16	243.6	
	40	277.5	138%	35-1R	0	201.2	
	44	282.3	140%		4	186.2	
					8	203.6	
					12	222.9	
					16	237.2	

Animal	Day Post Tx	weight	% of zero wt.	Animal	Day Post Tx	weight	% of zero wt.
29-0	0	210.8	100%	43-0	0	203.3	100%
	4	209.3	99%		4	185.6	91%
	8	215	102%		8	215	106%
	12	224.4	106%		12	228.8	113%
	16	235.2	112%		16	239	118%
	20	241.8	115%		20	259.5	128%
	24	252.7	120%		24	264.1	130%
	28	258.6	123%		28	275.1	135%
	32	262.9	125%		32	275.7	136%
	36	261.7	124%		36	283.3	139%
	40	242.3	115%		40	291.2	143%
	44	203.6	97%		44	299.4	147%
43-1R	0	199.5	100%	44-1R	0	205.6	100%
	4	175.4	88%		4	190.3	93%
	8	198.5	99%		8	217.8	106%
	12	213.8	107%		12	230.1	112%
	16	225.9	113%		16	237.8	116%
	20	241.6	121%		20	255.4	124%
	24	239.3	120%		24	255.9	124%
	28	249.3	125%		28	264.4	129%
	32	247.9	124%		32	263.2	128%
	36	258.3	129%		36	273.9	133%
	40	264.5	133%		40	277.4	135%
	44	275.9	138%		44	281.1	137%
30-1R	0	202.9		25-1L	0	211.6	
	4	174.8			4	172.4	
	8	DIED			8	DIED	
25-1R	0	217.7		36-0	0	210.1	
	4	186.2			4	181.2	
					8	204.9	
					12	223.2	
					16	239.5	
44-0	0	214.7		36-1R	0	212.2	
	4	191.8			4	187.3	
	8	212.3			8	213.1	
	12	222.3			12	223.8	
	16	239.4			16	239.2	

Appendix C- Percent Fibrosis Free Data

Material (animal)	% Free of Fibrosis after 4 days of implantation
PES (30-1R)	98.7
PES (25-1L)	91.7
PES (25-1R)	96.9
PTFE (41-1L)	94.0
PVC (24-1L)	96.4
PVC (24-1R)	97.2

Material (animal)	% Free of Fibrosis after 16 days of implantation
PES (36-0)	80.6
PES (44-0)	89.9
PES (36-1R)	77.8
PTFE (39-1L)	7.8
PTFE (39-0)	26.2
PTFE (39-1R)	22.5
PVDF (37-1R)	83.3
PVDF (37-0)	48.0
PVDF (35-1R)	73.0

Appendix C (Continued)

Material (animal)	% Free of Fibrosis after 44 days of implantation
PES (43-0)	96.4
PES (43-1R)	67.9
PES (44-1R)	37.8
PES (29-0)	85.7
PTFE (26-1R)	19.6
PTFE (30-1L)	19.7
PTFE (27-0)	43.0
PTFE (26-1L)	7.4
PTFE (41-1R)	23.6
PTFE (41-0)	59.1
PVC (29-1R)	98.4
PVC (42-0)	90.1
PVC (42-1R)	85.0
PVC (33-1L)	92.9
PVC (33-1R)	95.0
PVC (23-1R)	57.6
PVDF (28-1R)	84.8
PVDF (22-2R)	47.8
PVDF (22-1L)	69.9
PVDF (35-0)	73.0
PVDF (34-0)	72.2
PVDF (34-1R)	97.2
PVDF (27-1R)	61.4

Example Calculation: PTFE (41-1R)

Pouch Length: 4.8cm Pouch Width: 3cm

Total Surface Area: 4.8cm X 3cm = 14.4cm^2 (simplify into rectangular shape)

Radius of Large Pores: .2cm

Area of each Pore (πr^2): $(\pi)(.2)^2 = .13cm^2$

Number of Large Pores NOT covered with fibrosis: 3

Surface Area Free of Fibrosis: 1cm x 3cm = 3cm^2

$+ \underline{3(.13cm^2) = .38cm^2}$

$3.4cm^2$

Percent Free of Fibrosis: $(3.4cm^2/ 14.4cm^2) \times 100 = \underline{23.6\%}$

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